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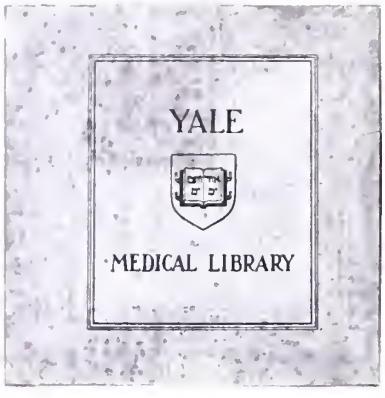
MODIFICATION OF THE MYOINTIMAL RESPONSE TO ARTERIAL INJURY: THE EFFECTS OF ASPIRIN AND WARFARIN

David Allen August

1980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MODIFICATION OF THE MYOINTIMAL RESPONSE TO ARTERIAL INJURY: THE EFFECTS OF ASPIRIN AND WARFARIN

David Allen August, B.S.

Massachusetts Institute of Technology

1980

A thesis submitted to the Yale University School of Medicine in partial fulfillment of the requirements for the degree of Doctor of Medicine

To my parents, Dr. Carola Eisenberg, and Dr. Martin Koplewitz, in gratitude for their inspiration as role models.

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INTRODUCTION

The human and economic impact of atherosclerotic vascular disease is formidable. The National Center for Health Statistics estimates that in 1978 almost 730,000 deaths, 38% of all deaths occurring in the United States that year, were attributable to atherosclerotic vascular disease or its direct complications (ischemic heart disease, cerebrovascular thrombosis, and cerebral embolism)[1]. The American Heart Association estimated the cost of cardiovascular disease in the United States to be approximately 28.5 billion dollars in 1978[62], and it is recognized that atherosclerosis accounts for more than 60% of all cardiovascular disease[87]. Yet despite the intense effort and vast amount of money expended on research related to atherosclerotic disease there has been frustratingly meager advancement toward the statement of a comprehensive theoretical framework within which clinical and basic science questions concerning atherosclerosis may be addressed. However, in the past decade primarily due to the work of Ross and Glomset and their colleagues in formulating and developing the response-to-injury hypothesis[44,62,88-91], progress toward the statement of a synthetic theory has been made. Already the response-to-injury hypothesis has permitted a more logical approach to major questions concerning the pathogenesis and progression of atherosclerosis such as the role of various risk factors, the natural history of vascular lesions, the origin and fate of incorporated lipids, the role of thrombosis in the disease process, the significance of the vascular smooth muscle cell, and the most promising modalities of prevention and treatment.

This study presents an overview of the development of the response-to-injury hypothesis, offers an up-to-date formulation of the patho-

physiological mechanisms postulated by this theory, and presents data collected during the course of my thesis research relevant to questions posed by this theory.

A MODERN THEORY OF THE PATHOGENESIS OF ATHEROSCLEROSIS

A. Background

All modern theories of the pathogenesis of atherosclerosis find their origins in the nineteenth century works of von Rokitansky and Virchow. In 1852 von Rokitansky proposed that:

> "The [atherosclerotic] deposit cannot be regarded as a product (exudation) of an inflammation of the arteries... The deposit is an endogenous product derived from the blood and for the most part from fibrin of the arterial blood."[86]

He postulated that through "metamorphosis" and degeneration these deposits evolve into advanced atherosclerotic plaques. In contrast Virchow viewed atheroma as the result of processes intrinsic to the vessel wall[100]. He claimed that irritation of the vascular endothelium results in a breakdown of the endothelial barrier, allowing infiltration of plasma constituents into the vessel wall. These insudated substances invoke a proliferative reaction of intimal connective tissue cells; reactive fibrosis in the overlying endothelium manifests itself as a fibrous cap covering the lesion. Mallory's demonstration of fibrin-like material in atherosclerotic lesions[61] and Clarke et. al.'s study displaying a spectrum of lesions ranging from simple mural thrombi to endothelialized clot-containing fibrous plaques[15] gave further credence to the significance of thrombosis in the development of atherosclerosis. However, it wasn't until the 1940's that Duguid refocused attention on the thrombotic mechanisms proposed by von Rokitansky. Invoking hemodynamic arguments he refuted Virchow's then popular view of atheroma as arising from fibrous overgrowth of degenerative fatty medial lesions[26] and cited his own work which demonstrated endothelialization of mural thrombi and their subsequent organization into intimal plaques[24,25] as

evidence for the crucial role of thrombosis in the genesis of atherosclerosis[26,27]. Investigations over the next twenty years[49,66], especially those looking at the role of fibrin deposition in the development of lesions[47,70], intimal thrombosis[39,53,76,80], and a thromboembolic rabbit model of atherosclerosis[42] reinforced the importance of thrombotic mechanisms in the inception and progression of atherosclerotic plaques. Concurrently, French emphasized the importance of endothelial structure and response[29]. Meanwhile Virchow's view of a proliferative medial response to intimal irritation was reawakened through studies[30,31,82-85,95] demonstrating that endothelial injury or loss results in the growth of fibrous intimal plaques characterized by the proliferation of smooth muscle cells. These lesions are similar to the focal fibromuscular lesions seen in human atherosclerosis[38,48,63]. However, none of these experimental lesions evidenced the accumulation or incorporation of lipid, except in the presence of hypercholesterolemia[84]. Renewed interest in the smooth muscle cell response to endothelial injury developed with the recognition that delayed re-endothelialization[10] or repeated endothelial injury[64,65] could lead to the development of lipid containing lesions in normolipemic animals. These findings stimulated further investigation which showed that endothelial injury is not merely an experimentally induced phenomenon but that it is an ongoing event in both animals[9,12,53,80,102] and man[46,53], and the sites where such injury is most often seen correspond to common loci of atherosclerotic disease.

In 1973 Ross and Glomset reviewed the role of the smooth muscle cell in atherosclerosis, emphasizing that focal intimal accumulation of these cells is fundamental to the entire process of atherogenesis[88]. Citing much of the evidence noted above, they highlighted the association of

endothelial injury with smooth muscle cell proliferation and the development of atherosclerosis. To explain this association they postulated that "the intact arterial endothelium normally acts as a barrier to some substance or substances present in plasma which upon exposure to vascular smooth muscle promote cell proliferation". They made no mention of a possible relationship of thrombotic processes to smooth muscle cell proliferation. It was not until the following year, with the identification of a platelet dependent serum factor mitogenic for smooth muscle cells[91] that the response-to-injury hypothesis emerged.

B. The Response-to-Injury Hypothesis[44,87-90]

Endothelial Injury (Figure 1)

Ross and Glomset posit intimal injury and the resultant focal endothelial desquamation as the initial events in atherogenesis. It is well established that endothelial injury or denudation resulting from almost any insult[10,14,28,30,45,81,82,85,94] will lead to the formation of fibrous plaques characterized by smooth muscle cell proliferation. That focal endothelial injury is a common phenomenon in animals and humans is also well documented[9,12,46,53,102]. Numerous factors have been identified as injury causing agents, including the interaction of hemodynamic forces and formed blood elements with the vessel wall[36,40], hypertension[36,40], microbiologic agents[11,22,27], immunologic agents[55], blood borne carbon monoxide derived from cigarette smoke and air pollution[98], lipids and cholesterol[3,50,52,78,92], thrombin[60], and inappropriate concentrations of normal biologic metabolites[45]. Moore has reviewed an extensive body of data and concluded that these factors are indeed operational in humans[67]. He notes the frequent occurrence of atherosclerotic lesions in human situations where these factors can

be implicated, including: (1) the association of atherosclerosis with the risk factors of hypertension, hyperlipidemia, and cigarette smoking; (2) atherosclerotic lesions occurring in the walls of autologous vein grafts exposed to the relative hypertension of arterial pressure; (3) the occurrence of atherosclerosis in the arteries of transplanted organs which are constantly exposed to immunologic damage; (4) the high incidence of thromboembolic phenomena in homocystinurics; and (5) the frequent finding in autopsies performed on neonates who had intra-aortic catheters in place of atherosclerotic lesions near the location of the catheter tip. The final connection of endothelial injury to atherosclerosis is made by studies demonstrating that when the endothelial insult is repetitive or chronic the fibrous plaques will incorporate and retain lipids[10,33,64,65,67,92].

Platelets and Plasma Constituents (Figures 2 and 3)

Focal endothelial desquamation exposes subendothelial cells and connective tissue to platelets and other blood components. Ross and Glomset theorize that this blood-subendothelium interaction is responsible for the smooth muscle cell response to endothelial injury. Among blood constituents, lipoproteins[91], insulin[96], and various hormones[99] have been shown to stimulate the migration or proliferation of cells in vitro, and they may play a similar role in the in vivo stimulation of smooth muscle cells. Tissue thromboplastin released by damaged cells and collagen initiate the clotting cascade to generate thrombin[71]. Although mitogenic for cultured fibroblasts thrombin probably does not stimulate smooth cells directly. However thrombin can alter the endothelial surface so that platelets will adhere to it[13] and can stimulate platelet aggregation and degranulation[54].

The most important blood borne mediator of the vascular response to injury is the platelet. Platelets adhere to the exposed subendothelial matrix, and their interaction with collagen induces platelet degranulation[73]. Among the substances released by platelet degranulation is a platelet dependent smooth muscle cell mitogen (PDSMCM) that was first identified by Ross, et. al.[91] in 1974. The PDSMCM is a heat stable basic protein released from platelet alpha-granules during degranulation caused by thrombin, ADP, and other releasing agents. It is mitogenic for cultured smooth muscle cells[89,91]. The PDSMCM and perhaps other blood derived substances are presumed by Ross and Glomset to be the in vivo stimuli for smooth muscle cell migration and proliferation subsequent to endothelial injury[44,89-91]. The critical in vivo role of platelets in the vascular response to injury is confirmed by studies showing the inhibition of fibrous plaque formation by the induction of thrombocytopenia[35,68] or treatment with the anti-platelet drug dipyridamole[45] prior to the causation of endothelial injury.

Progression or Regression? (Figures 4 and 5)

The response-to-injury hypothesis posits two alternative pathways for the evolution of an intimal injury. If the developing plaque is permitted to re-endothelialize regression of the lesion occurs (Figure 4). Such regression is observed in many animal models involving a limited endothelial injury[8,14,28,34,82,83,94,95]. However, repeated or chronic bouts of endothelial injury can lead to continuing smooth muscle cell proliferation with lipid incorporation* (Figure 5); such

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* One can even envision a scenario whereby the initial injury and resulting smooth muscle cell proliferation, by creating a raised plaque protruding into the vessel lumen which is therefore subject to greater hemodynamic stresses, actually predisposes to further injury.

a progression is seen in a variety of animal models[33,64,65,67]. Continued proliferation and lipid incorporation may also occur when endothelial injury synergizes with hyperlipidemia[2,43,69,77]. The synergistic effect of hyperlipidemia may be due to a combination of factors including a direct stimulatory effect of lipids on smooth muscle cells[88,91], an injurious effect of lipids on endothelium[3,50,52,78,92], lipid induced platelet hypersensitivity[20,57,75] (cigarette smoking also causes platelet hypersensitivity[75]), and an irritative effect of insoluble insudated cholesterol[88]. Lipid accumulating in the lesion may be derived from either insudated serum lipids or from the smooth muscle cells themselves; this process is not well understood[89]. Regardless, once a lipid containing plaque is established the well characterized processes of thrombosis and embolization can then act to lead to the development of complicated atherosclerotic plaques and overt clinical sequelae[25,27,42,73,74].

Summarizing, the response-to-injury hypothesis postulates endothelial injury as the initiating factor in atherogenesis, smooth muscle cell proliferation and migration in response to the PDSMCM and other serum factors as the initial vascular response, and either regression of the proliferative lesion or progression in the face of continuing intimal insult to an atherosclerotic plaque. The theory accounts for many experimental and clinical observations concerning atherosclerosis, including the role of endothelial injury, the significance of smooth muscle cell proliferation, the importance of platelets and thrombosis, and the association of certain risk factors with clinical disease patterns. As Ross and Glomset are quick to note however, there are gaps in the hypothesis. It does not in its present form answer questions regarding the differential incidence and severity

of atherosclerosis among individuals and population groups. The mechanisms of plaque regression, lipid incorporation, and other details concerning the role of lipids are not specified. The significance of the observations of Benditt and Benditt suggesting that the proliferation of smooth muscle cells is monoclonal[6,7] is not addressed. Many details of the theory also remain to be elucidated, including a further characterization of the biology and surface characteristics of the smooth muscle cells, a more complete characterization of the PDSMCM and other possible mitogens, the role of thrombin and other coagulation factors in the response to injury, determination of the detailed mechanism of platelet function at the injury site, and the identification of pharmacological agents that may be used to selectively alter the response to injury in both research and clinical settings.

C. This Investigation

The objectives of this study are threefold: (1) to confirm the validity of a model recently developed to investigate the vascular response to endothelial denudation in the rat; (2) to further characterize observations made with this model; and (3) to use this model to obtain new data regarding the role of thrombin in the response to intimal injury.

The use of this model was first reported by Fishman, et. al.[28]; they adapted it from a technique employed to study the injury response of peritoneal mesothelium[93] and have since used the model extensively[16-19]. It involves induction of endothelial desquamation and resultant fibrous plaque formation through creation of a desiccating injury in the rat carotid artery. This model was selected for use in this investigation for several reasons: (1) as a potentially valuable technique for

the study of myointimal response to vascular injury it warrant further characterization; (2) the endothelial denudation produced is complete and occurs in a well defined segment of artery; (3) in contrast to other techniques used to create intimal injury[10,14,30,31,46,64,65,81-85,94,95,97] there is no metabolic or mechanical insult[28,51,94] to the media; the injury is purely endothelial; (4) the rat is a well characterized and inexpensive experimental model; and (5) pharmacologic intervention in rats is relatively simple.

The effect of aspirin on the vascular response to injury was investigated because of its well known effects on platelets[101] and because of the attention it has received as a possible treatment and/or prophylaxis for clinical cardiovascular disease[72]. This aspect of the study repeats investigations made elsewhere[5,18].

Interest in the role of thrombin in the myointimal response to injury suggested that the effect of an anti-thrombin agent on the model be studied. Heparin has previously been shown to blunt the proliferative response to injury and it was speculated that this effect may be due to heparin inhibition of thrombin formation[17]. However, the pharmacological activities of heparin are complex and varied. To more clearly elucidate the role of thrombin in the injury response the effect of warfarin, a drug whose effects are limited solely to its inhibition of synthesis of the vitamin K dependent clotting factors (including prothrombin)[58], was investigated.

METHODS

Fifty-eight male Sprague-Dawley rats, each weighing 175 to 250 grams, were obtained from Camm Research Institute (Wayne, New Jersey). All rats were allowed free access to Purina Rodent Laboratory Chow 5001 (Ralston Purina Company, St. Louis, Missouri) and water at all times. Assignments to experimental groups were made randomly. Twenty rats were assigned to the control group, fourteen to the aspirin group, and twenty-four to the warfarin group.

Operation

All operations were carried out under ether anesthesia and involved the creation of a desiccating injury to the left common carotid artery endothelium according to the method of Fishman, et. al.[28]. Briefly (see Appendix for a detailed 'op-note'), the procedure involved exposure of a 2.5 - 3.5 cm. unbranched segment of left common carotid artery through a midline cervical incision. Two 4-0 silk ligatures were tied tightly around the artery approximately 2 cm. apart. A 30-gauge needle attached via a connecting tube and three-way stopcock to a syringe containing normal saline and a syringe containing 60 ml. of room air was then inserted into the lumen of the isolated arterial segment just distal to the proximal ligature and passed along the lumen, carefully avoiding contact with the vessel wall. The needle was advanced to a point just proximal to the distal ligature where it was allowed to penetrate the full thickness of the vessel wall. It was then withdrawn back through the lumen until only the tip remained intraluminal and used to rinse the isolated segment with approximately 0.5 ml. of saline. Next the stopcock valve was switched and the 60 ml.

of air was streamed gently through the lumen at a rate of 30 ml. per minute to produce the desiccating endothelial injury. The lumen was then refilled with normal saline and the ligatures removed. Hemostasis was achieved with gentle pressure applied to the needle holes with dry or saline moistened gauze sponges; hemostasis was generally attained in less than three minutes in the control and aspirin treated rats but often required 7-10 minutes in the warfarin treated rats. The skin incision was closed with a single layer of interrupted 4-0 silk sutures.

Carotid artery specimens for histological examination were obtained via re-operation through the original incision under ether anesthesia. The left common carotid artery was again isolated, 4-0 silk ligatures placed proximally and distally, and the isolated (previously desiccated) segment resected using a small dissecting scissors and small blunt forceps. The sample was immediately rinsed in normal saline and placed in formalin. With the rat still alive a 2-3 ml. blood sample was then obtained via cardiac puncture with a 23-gauge needle for clotting time determination.

Pharmacological Protocol

Control group rats received no pre-operative or post-operative treatment. Two rats were sacrificed without undergoing the desiccation procedure (Day 0) and two each were sacrificed on the first, third, fifth, seventh, tenth, twenty-first, and twenty-eighth post-operative days. Three control rats were sacrificed on the fourteenth post-operative day. (Table 1)

The aspirin treated rats received 160 mg. per kg. of powdered aspirin (McKesson) suspended in glycerin U.S.P. to a concentration of

50 mg. per ml. The aspirin suspension was administered via gavage through a 16-gauge dosing needle 24 hours pre-operatively, immediately pre-operatively, and then each morning post-operatively until sacrifice. Three rats were sacrificed on each of the post-operative days 7, 14, 21 and 28. (Table 1)

The warfarin group rats received 0.1 mg. per kg. of warfarin (Endo) dissolved in tap water to a concentration of 0.05 mg. per ml. and administered via gavage through a 16-gauge dosing needle. The rats were dosed 12 hours pre-operatively, immediately pre-operatively, 36 hours post-operatively, and then each morning until sacrifice. Two rats were sacrificed on the seventh post-operative day, and three each on the fourteenth and twenty-eighth post-operative days. (Table 1)

Clotting Times

Clotting times were determined from 1 ml. samples of whole blood according to the method of Lee and White[56] to the nearest half-minute. Control samples were obtained from age and weight matched male Sprague-Dawley rats obtained from Camm Research Institute.

Microscopy

Tissue specimens for light microscopy were fixed in formalin for at least 48 hours, embedded in paraffin, and sectioned. Sections were obtained from two to four longitudinally arrayed locations along the injured segment of artery. Sections were stained with hematoxylin and eosin or according to the Elastin-Van Giemsa method.

Intima:media (I/M) ratios were determined by recording for each section of each specimen the maximal ratio of intimal thickness (distance from the luminal surface to the internal elastic lamina) to medial thickness (distance from the internal to the most external

elastic lamina) as measured with an optical graticule, and averaging these ratios for the sections to arrive at an average I/M ratio for each particular specimen. For each sample day a weighted average of these specimen averages ± S.E.M. was calculated. (All I/M ratios ± S.E.M. cited in this text are x 100.) Sections were scored only if there was no evidence of luminal or mural thrombosis, the boundaries of the intima and media were clearly demarcated, and the internal elastic lamina was intact. Control and experimental group means were compared using Student's t-test and hyperplasia growth rates were compared via linear regression analysis.

RESULTS

Of the 20 control group rats one developed thrombosis of its left common carotid artery and was discarded. All control group rats gained weight and thrived. One of the 14 aspirin group rats died for no apparent reason on the third post-operative day and one developed thrombosis of its left common carotid artery; all others gained weight, thrived, and completed the study. Sixteen of the 24 rats in the warfarin group died in the period two to nine days post-operatively, apparently secondary to bleeding diatheses. Of the eight animals surviving to completion of the study four evidenced some weight loss and four demonstrated weight gain and thrived.

Clotting Times

The clotting times of the control rats and the aspirin treated rats did not differ significantly (Table 2). The warfarin dosage used in this study was sufficient to significantly prolong the clotting time of the warfarin treated rats in comparison to those of both the control group (2.8 ± 0.5 min. vs. 1.3 ± 0.2 min.; $p < 0.05$) and the aspirin group (2.8 ± 0.5 min. vs. 1.5 ± 0.1 min.; $p < 0.05$) (Table 2).

Histology

Control Group - The observations confirmed those reported by Fishman, et. al.[28]. Pre-operatively (Photograph 1) there is a monolayer of delicate endothelial cells with elongated nuclei oriented parallel to the vessel wall overlying a densely populated dormant appearing media. At Day 1 post-operatively (Photograph 2) the endothelium is completely denuded exposing the internal elastic lamina. The innermost layers of the media evidence some edema. At Days 3 and

5 the medial edema has increased to encompass the full thickness of the media giving it a vacuolated appearance in both the hematoxylin and eosin and the Elastin-Van Giesma slides. Occasional medial mitoses are seen. There are no endothelial cells visible in Day 3 slides but by Day 5 endothelial regeneration is evidenced by the appearance of an occasional randomly oriented endothelial cell with a swollen nucleus. Occasional disruptions in the internal elastic lamina are noted, probably secondary to needle trauma.

Day 7 marks the onset of myointimal proliferation. Layers of swollen endothelium two to four cell layers thick now cover many portions of the formerly exposed internal elastic lamina. In some areas proliferating myointimal cells insinuate themselves between the endothelium and the internal elastic lamina. Medial edema persists and mitoses are still observed in the media. Over the following fourteen days (Photographs 3-5) myointimal and endothelial proliferation continue, peaking sometime between Days 10 and 21. In many specimens myointimal proliferation is the predominant feature, occasionally creating plaques 20 to 30 cells layers thick coated by swollen endothelial cells. This proliferation is not uniform in transverse sections of vessels, leaving some areas untouched (Photograph 8), but it is regular along the length of the vessel. Elastin-Van Giesma stained slides show the plaques to be virtually overrun with elastin fibers. During this period the medial edema begins to resolve and mitoses become infrequent. By Day 28 (Photograph 6) the hyperplastic myointima is beginning to recede resulting in a decrease in the average I/M ratio. Most of the medial edema has resolved.

At no time during this process is there evidence of medial in-

filtration or adherence to the denuded internal elastic lamina by polymorphonuclear leukocytes or other inflammatory cells. Myointimal hyperplasia is often most pronounced where the internal elastic lamina is interrupted, but these sites were not used in the determination of I/M ratios.

Aspirin Group - The observations confirm those of Clowes, et. al.[18]. They differed from the observations made on the control specimens in only two respects: 1) the maximal myointimal response is not seen until Day 21 (Photographs 7-9), somewhat later than in the control group; and 2) in three of the aspirin treated rats (one sacrificed on Day 21 and two on Day 28) very localized areas of striking myointimal proliferation occurs giving the appearance of intraluminal polyps (Photographs 10 and 11). These "polyps" are endothelial coated outgrowths of myointima, occasionally growing in whorled patterns, resting on intact internal elastic laminae. The myointimal cells are benign in appearance.

Warfarin Group - Throughout the observation period the changes in the media of warfarin treated rats parallel those seen in control group rats; edema is evident but no inflammatory infiltrate is noted. However, differences in endothelial regeneration and myointimal proliferation are noted. Regenerating endothelium in the warfarin group rats resembled normal endothelium, for the most part lacking the swelling and disorientation seen in the control and aspirin groups. Furthermore, although the peak I/M ratio in the warfarin treated rats is no different from that seen in the control group (Photographs 12 and 13), the hyperplastic myointima resolves more quickly in the warfarin treated group (Photographs 14 and 15), resulting in a significantly lower I/M ratio

in Day 28 warfarin group rats than in Day 28 control rats (16.2 ± 2.2 vs. 26.0 ± 3.3 respectively, $p < 0.05$).

Intima/Media Ratios

The I/M ratios observed are summarized in Table 3. Comparisons of the control group data with the aspirin group and warfarin group data are made in Graphs 1 and 2 respectively. The hyperplasia in the control group is seen to peak on Day 10 at an I/M value of 77.8 ± 21.0. The aspirin treated group did not achieve its peak I/M value until Day 21, when it was 130.2 ± 50.5. Maximum hyperplasia was observed in the warfarin group on Day 14 with an I/M ratio of 44.5 ± 26.8.

These data confirm the histologic impression of the occurrence of significant hyperplasia in the control and aspirin groups. At all times beyond Day 6 the I/M ratios seen in control and aspirin group rats differ significantly from that seen in unoperated controls ($p < 0.05$; Table 4). No significant difference can be demonstrated between the I/M ratio observed in unoperated controls and the warfarin group at Days 7 and 14, although a statistically significant difference is noted at Day 28 (16.2 ± 2.2 vs. 8.7 ± 1.5; $p < 0.05$; Table 4).

These findings also confirm the observations of Clowes, et. al.[18] that aspirin does not affect the rat carotid artery myointimal response to desiccating endothelial injury. There are no significant differences in the I/M ratios at corresponding times between the control and aspirin groups (Table 3, Graph 1). Linear regression analysis reveals no significant difference in the rate of increase of the I/M ratios in the control and aspirin groups.

There is no significant difference between the I/M ratios determined in the control and warfarin groups at Days 7 and 14 but there is

a statistically significant greater degree of hyperplasia in the control group than in the warfarin group at Day 28 (26.0 ± 3.3 vs. 16.2 ± 2.2; $p < 0.05$; Table 3, Graph 2). Again linear regression analysis reveals no significant difference in the rate of growth of the I/M ratios in the control and warfarin groups.

DISCUSSION

Under these experimental conditions carotid artery endothelial denudation leads to myointimal thickening characterized by smooth muscle cell proliferation and elaboration of connective tissue. This finding concurs with those of numerous other investigators who have studied this response. The histologic appearance and evolution of the lesions is also similar to that reported by other groups[10,14,28,30,45,81,82,94,95]. In particular this study confirms results obtained by Clowes et. al.[78] using this same experimental model.

The chronology of development of the features observed in this study is quite similar to that previously reported[28]. By twenty-four hours after desiccation endothelial denudation is uniformly complete and medial edema is apparent. Day 7 marks the onset of myointimal hyperplasia by light microscopic criteria and frequent medial smooth muscle cell mitoses are evident. Peak myointimal thickening is observed near Day 14. Throughout the period of observation, for reasons not apparent, the myointimal response is noted to be circumferentially irregular. In contrast to observations previously reported[18,28], in the current study medial edema persists through Day 28 and the regenerating endothelial cells lack the usually well-defined nuclear orientation seen in undisturbed endothelium. These differences are not deemed conceptually significant. For no apparent reason the magnitude of the I/M ratios observed in this study in all groups is less than those reported by Clowes et. al.[18,28] by a factor of approximately ten.

Observations made on the aspirin treated rats also confirm those

made elsewhere[18], again with the exceptions of the persistence of medial edema and the irregular orientation of endothelial cell nuclei. Additional observations made in the aspirin group in the course of this study include: (1) delay of the peak myointimal response until Day 21, but because of the marked biological variability seen it is difficult to attribute this to an effect of aspirin; and (2) the formation of benign polypoid outgrowths of neointima seen only in aspirin treated rats; their significance is not known.

The warfarin treated rats exhibit several important deviations from the findings seen in untreated rats. No statistically significant difference can be demonstrated between the I/M ratios observed in Day 7 and Day 14 warfarin group rats and unoperated control group rats, although there are clear histologic differences; and the magnitude of myointimal hyperplasia seen at Day 28 in warfarin treated rats is statistically significantly less than that seen in the control group rats. Both of these findings indicate that warfarin has a suppressive effect on the smooth muscle cell response to endothelial injury.

The absence of an aspirin effect on the myointimal response to vessel wall injury is in agreement with previous reports on rat[18] and rabbit[5] models. It is understandable in light of current concepts regarding the mechanisms of platelet interaction with denuded vessel wall and the pharmacologic actions of aspirin. As summarized by Mustard, et. al.[73,74] endothelial injury results in platelet adherence to damaged endothelium and exposed subendothelial structures. Subendothelial collagen stimulates platelet granule release and in conjunction with tissue thromboplastin derived from damaged cells activates the coagulation cascade. Generated thrombin, platelet

released ADP, and endoperoxides PGG_2 and PGH_2 and thromboxane A_2 formed via the arachidonate pathway cause additional platelet adherence, recruitment of blood borne platelets (aggregation), and platelet granule release. Platelet adherence to collagen and collagen stimulated platelet degranulation are independent of the arachidonate pathway[13]. Collagen dependent aggregation is in part mediated via the arachidonate pathway[13]. Platelet degranulation results in the release of the PDSMCM[89,91].

It has been shown that after the initial adherence of a platelet monolayer to denuded vessel wall little subsequent platelet turnover or aggregation occur[41] and fibrin formation is minimal[4], indicating that the critical events are the initial events of adherence and degranulation. This concept receives further support from a study showing that to inhibit fibrous plaque formation thrombocytopenia must be induced <u>prior</u> to endothelial injury[32]. Aspirin affects thrombus formation by blocking the formation of PGG_2, PGH_2, and thromboxane A_2, through the arachidonate pathway[74]. Therefore while aspirin inhibits platelet aggregation it does not affect platelet adherence to subendothelial structures or platelet degranulation. Thus it has no effect on the processes felt to be necessary for release of the PDSMCM. The expectation that a drug which inhibits platelet adherence and degranulation should reduce the extent of fibrous plaque formation is confirmed by a study looking at the effect of dipyridamole in homocystinemic baboons[45].

The effect of warfarin on the vessel response to injury is almost certainly related to its suppression of the synthesis of the vitamin K dependent clotting factors (prothrombin, VII, IX, and X), as it is

largely devoid of other pharmacological activity[58]. In particular, its suppressive effect is probably mediated through its inhibition of thrombin formation. However, the details of this effect can only be conjectured as the role of blood coagulation in the response of the vessel wall to injury is not well defined.

It is known that thrombin stimulates platelet aggregation and degranulation through mechanisms at least partly independent of ADP and the arachidonate pathway[79]. Thrombin induced degranulation causes release of the PDSMCM[91]. Additionally, exposure of intact endothelium to thrombin alters it such that it will permit adhesion of platelets[13]. Thus, within the framework of the response-to-injury hypothesis, factors which inhibit thrombin formation might be expected to blunt the smooth muscle cell response by: (1) inhibiting the thrombin mediated pathway that leads to release of the PDSMCM; and (2) inhibiting thrombin induced endothelial injury and the resultant platelet adhesion.

In support of the importance of thrombin to the smooth muscle cell response to injury is evidence showing marked suppression of the response by heparin[17]. However, it is impossible to determine from that study whether the suppression is a result of the antithrombin activity of heparin or one of its many other biological actions. As a strong cation heparin may bind the basic PDSMCM[17]. Heparin inhibits wound healing in a variety of situations and may somehow alter the smooth muscle cell response through a similar effect on endothelial injury[17]. The binding of heparin to negatively charged subendothelial structures[23] may alter platelet-subendothelium interactions, although this is not morphologically apparent[17]. Heparin may similarly bind to the negatively charged platelet surface[23] and inhibit platelet surface

interactions. Lipoprotein lipase, a vessel wall associated enzyme that clears chylomicrons and fatty acids from blood and that decreases platelet adhesiveness is released by heparin and may thus influence both platelet activity and local lipid effects[58].

Because the only pharmacologic action shared by heparin and warfarin is inhibition of thrombin formation it is reasonable to speculate that their similar suppressive effect on the vascular response to intimal injury is mediated by this anti-thrombin activity. Of course the possibility that warfarin exerts its effect solely through its inhibition of the synthesis of clotting factors VII, IX, and X and that the heparin suppression is independent of its anti-thrombin activity cannot be excluded at this time. If indeed both drugs do act through an anti-thrombin mechanism, this indicates a central role for thrombin in the response-to-injury hypothesis. Thrombin may exert its influence through some combination of its effects on endothelium and platelet function. More speculatively, the demonstration of the mitogenicity of the PDSMCM for smooth muscle cells was carried out in the presence of thrombin[91] and perhaps the proteolytic action of thrombin is necessary to activate the PDSMCM or to alter smooth muscle cell surface receptors so as to make them more receptive to the mitogen.

SUMMARY

This study used a rat model of desiccating carotid artery endothelial injury to examine the vessel response to endothelial denudation and to look at the effect of aspirin and warfarin on this response. While aspirin had no apparent effect warfarin reduced the magnitude of the myointimal response. It is conjectured that this is indicative of an important but as yet undetermined role for thrombin in vascular response to injury. This has important implications for the role of thrombin in the response-to-injury hypothesis of atherogenesis.

APPENDIX: OPERATIVE NOTE

After anesthetization to unresponsiveness in an ether chamber the rat was placed on a wooden surgical board and secured by its extremities. A rolled up 4 x 4 gauze was placed under the animal's neck to attain cervical extension. Anesthesia was maintained throughout the procedure with an ether soaked gauze sponge placed in a 50 ml. beaker near the rat's snout; anesthetic depth was regulated by varying the distance of the beaker from the snout and monitored by frequent observation of respiratory pattern and responsiveness to intra-operative stimuli, with special attention to frequently occurring laryngospasm.

With the animal properly positioned the anterior neck was shaved and a midline incision made with a #11 knife blade from a point just superior to the hyoid bone to a point approximately 1/2 cm. superior to the sternal notch. Using blunt and sharp dissecting techniques the fascia joining the submaxillary glands across the midline was divided; the submaxillary glands were freed from underlying attachments and retracted laterally under the tension of a pair of small hemostats. Next the sternohyoid muscles were divided longitudinally in the midline via blunt dissection and retracted with a small self-retaining retractor to expose the trachea. A small curved hemostat and a pair of small pointed forceps was used to locate the left common carotid artery by sliding along the left margin of the trachea (Photograph 17). An unbranched 3 cm. segment was freed up. Two 4-0 silk ligatures were placed around the artery such that they isolated a 2 cm. segment and were secured tightly with slip knots to allow atraumatic removal (Photograph 18).

Next, using a x2 magnifying visor and the proximal ligature to manipulate the artery, a 30-gauge needle attached to two 50 ml. syringes with a connecting tube and three-way stopcock (Photograph 19) was inserted into the proximal portion of the isolated segment, carefully passed up through the lumen, and then allowed to penetrate the full thickness of the vessel wall just proximal to the distal ligature. To prevent tearing the vessel this distal puncture was made while using surrounding muscle tissue to "back" the arterial wall and thus support it. The needle was then carefully withdrawn such that only its tip remained intraluminal (Photograph 20). The air filled syringe was depressed so that the 0.5 ml. of normal saline filling the connecting tube rinsed the lumen and then a gentle stream of air (30 ml. per minute) was passed through the lumen for two minutes to cause the desiccating injury. When this was completed the stopcock was switched to permit refilling of the lumen with normal saline from the saline syringe and the needle was fully withdrawn. The distal ligature was removed and hemostasis achieved with gentle pressure from a combination of moist and dry gauze sponges, and then the proximal ligature was removed with bleeding controlled similarly (Photograph 21).

Before closing the neck was rinsed thoroughly with normal saline and the sternohyoids were reapproximated with a non-absorbable 4-0 silk suture to assist anatomical orientation at the time of specimen recovery. The skin was closed with interrupted 4-0 silk sutures (Photograph 22). Approximately two thirds of the rats undergoing this procedure had a left Horner's Syndrome post-operatively, but otherwise apparently complete recovery was achieved within 24 hours.

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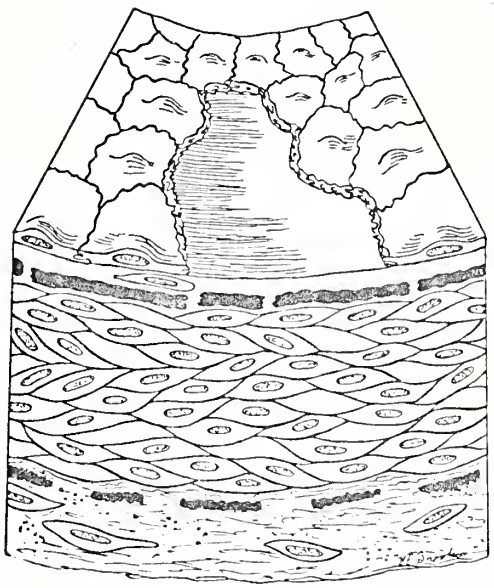


FIGURE 1

Endothelial injury, which may be caused by many different forms of injury, exposes the sub-endothelium to platelets and other blood components (89).

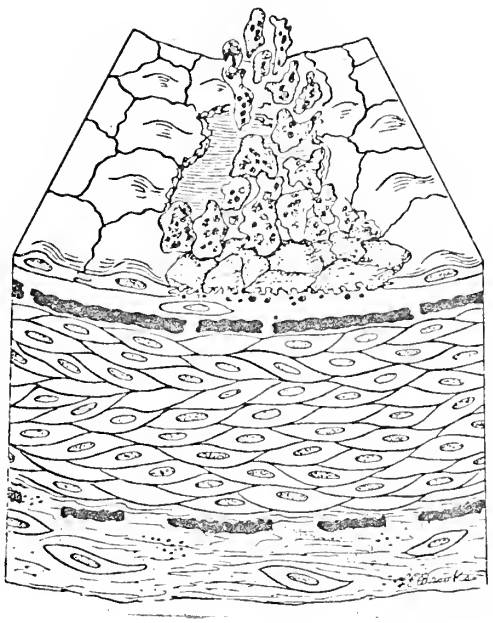


FIGURE 2

A monolayer of platelets adheres to the exposed subendothelium. Collagen in the sub-endothelium stimulates the platelets to degranulate, releasing a variety of factors including the platelet dependent smooth muscle cell mitogen (89).

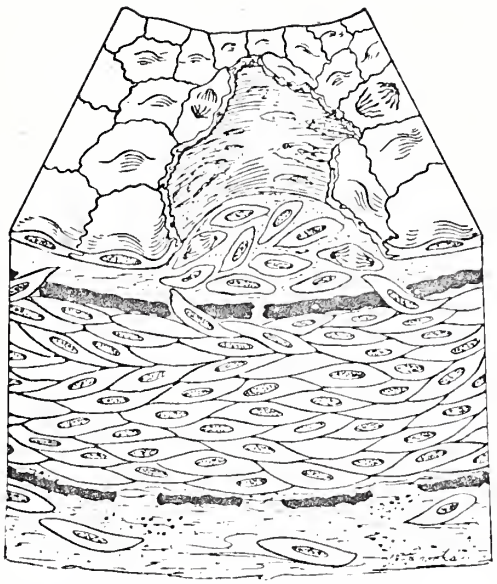


FIGURE 3

Smooth muscle cells migrate from the media into the intima through fenestrae in the internal elastic lamina and proliferate in response to platelet factors and other plasma constituents (89).

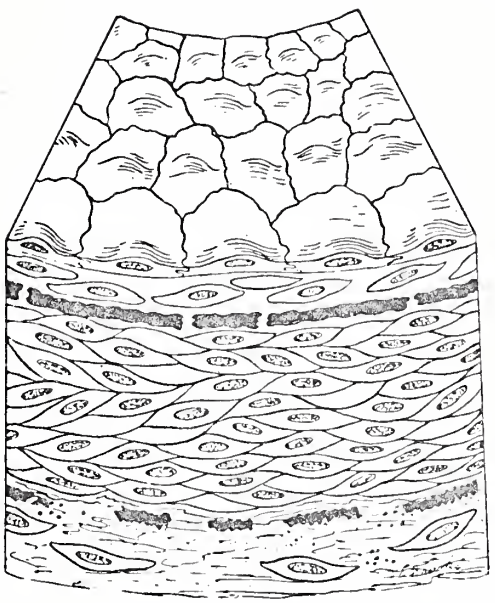


FIGURE 4

Regression of the proliferative lesion shown in Figure 3 occurs if the source of endothelial injury disappears, leaving a slightly thickened intima containing a few layers of smooth muscle cells (89).

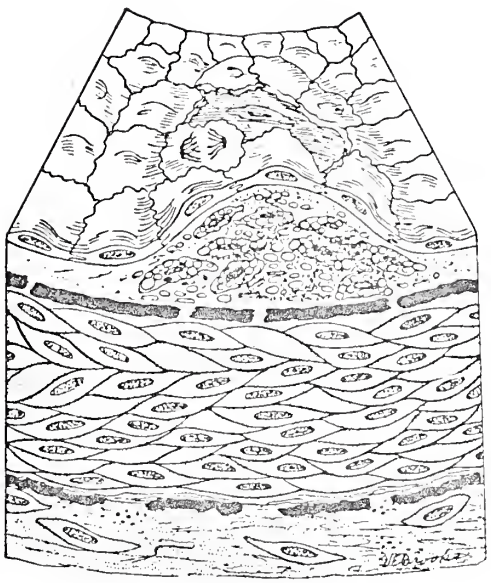


FIGURE 5

Progression of the proliferative lesion shown in Figure 3 occurs if the source of endothelial injury is chronic or recurrent, resulting in a proliferative lesion of cells and connective tissue in a pool of lipid. Thrombosis may cause further development into a complicated atherosclerotic plaque (89).

SACR:			2	2	2	2	2	3	2	2	2	2	
CONTROL			0										
			P										
SACR:			E					3		3	3	3	
ASPIRIN			R										
DOSE:	**		A	** 160 MG. PER KG. EACH MORNING									
			T										
SACR:			I					2		3		3	
WARFARIN													
			O										
DOSE:		****	**** N	**** 0.1 MG. PER KG. EACH MORNING									
TIME:	-24HRS.	-12HRS.	DAY 0	1	2	3	5	7	10	14	21	28	

TABLE 1

EXPERIMENTAL PROTOCOL: Aspirin and warfarin dosing schedules and number of animals sacrificed per day.

** - 160 mg/kg **** - 0.1 mg/kg

DRUG GROUP	CLOTTING TIME	NUMBER IN GROUP
CONTROL	1.3 ± 0.2	8
ASPIRIN (160 mg/kg/day)	1.5 ± 0.1	12
WARFARIN (0.1 mg/kg/day)	2.8 ± 0.5	8

TABLE 2

CLOTTING TIMES: Clotting time in minutes ± S.E.M. as measured by the method of Lee and White (56).

I/M RATIO x 100

DAY	CONTROL	ASPIRIN 160 mg/kg/day	WARFARIN 0.1 mg/kg/day
0	8.7 ± 1.5 (4)*	---	---
1	0.0 ± 0.0 (3)	---	---
3	13.6 (1)	---	---
5	12.2 ± 4.4 (4)	---	---
7	21.5 ± 1.5 (4)	17.4 ± 2.3 (5)	11.2 ± 3.2 (2)
10	77.8 ± 21.0 (6)	---	---
14	21.0 ± 4.6 (8)	33.8 ± 6.3 (12)	44.5 ± 26.8 (10)
21	34.8 ± 3.7 (6)	130.2 ± 50.5 (12)	---
28	26.0 ± 3.3 (5)	60.0 ± 18.1 (10)	16.2 ± 2.2 (8)

TABLE 3

INTIMA/MEDIA RATIOS: I/M ratios ± S.E.M. x 100 in injured carotid arteries at various time points. Day 0 is the day of operation. Day 0 control values are for uninjured vessels.

* - Number of data points in each group is in parentheses.

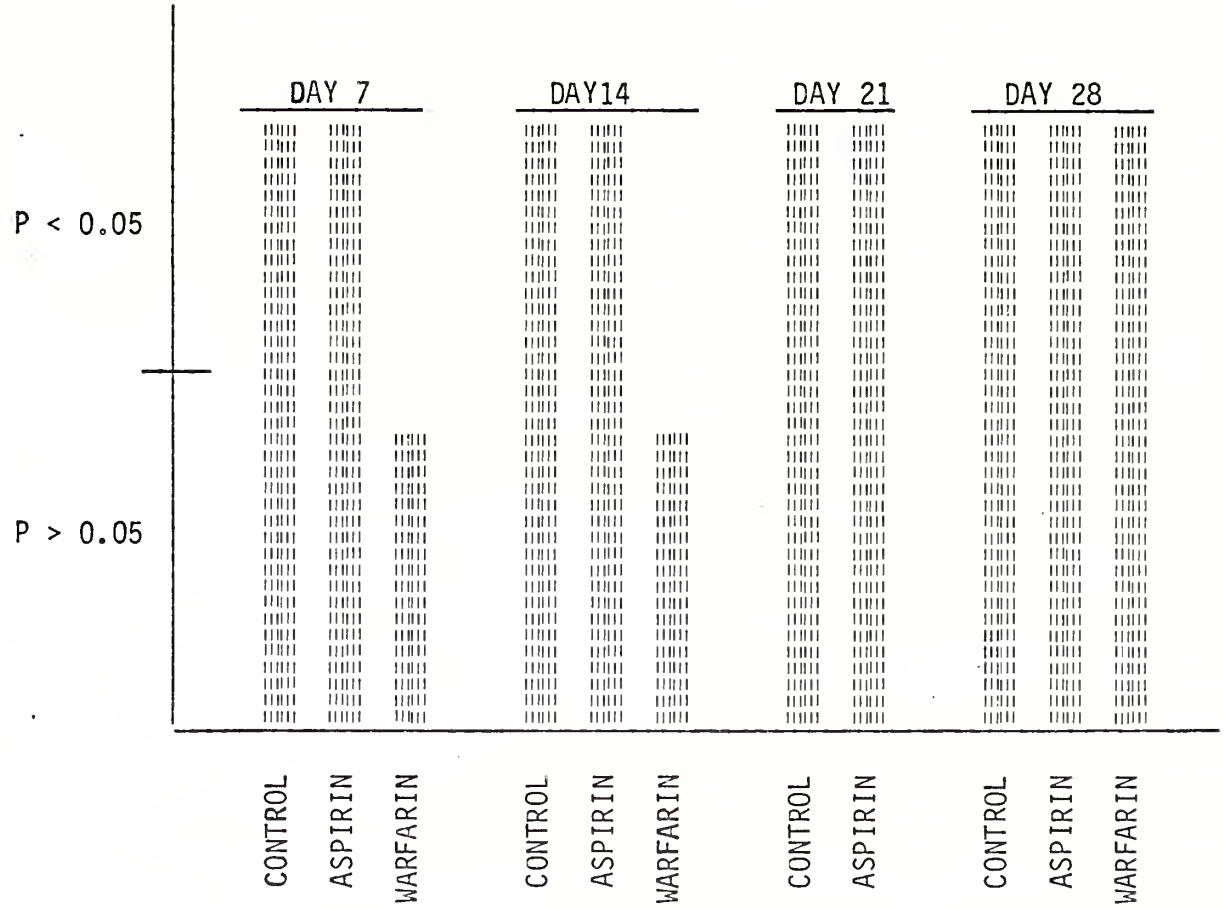
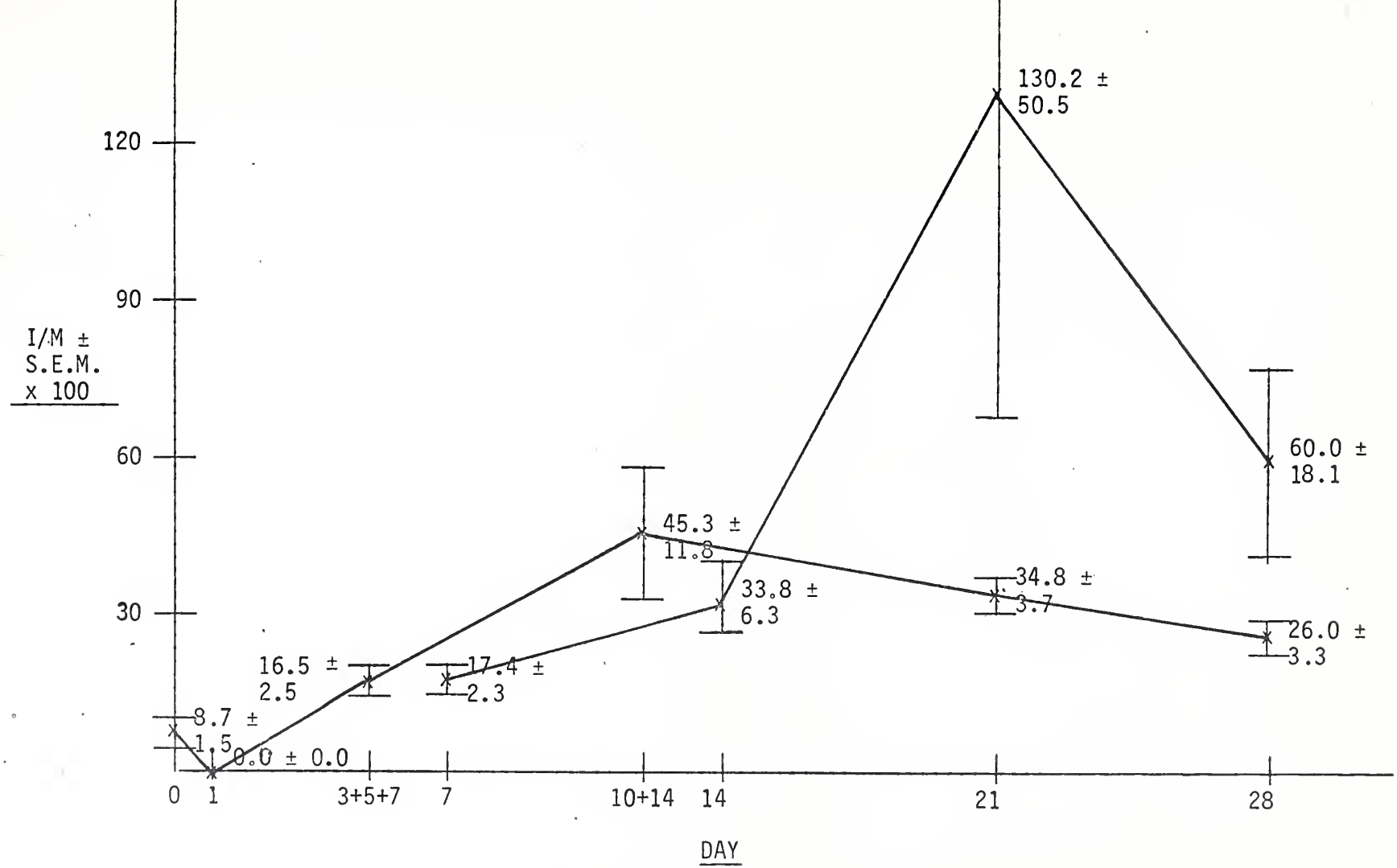


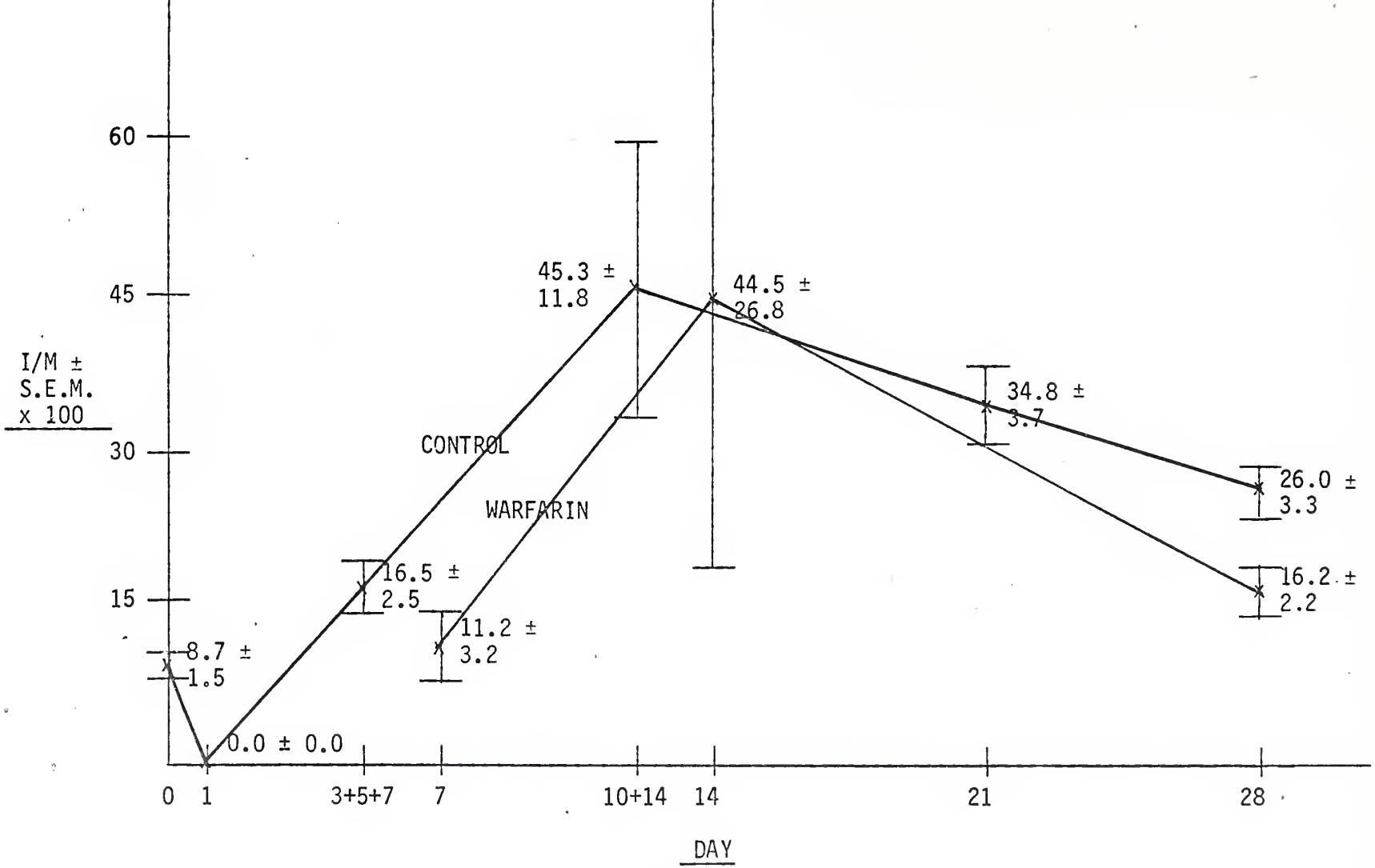
TABLE 4

CHANGE FROM DAY 0: I/M ratios compared with the I/M ratio in Day 0 unoperated controls using the Students' t-test.



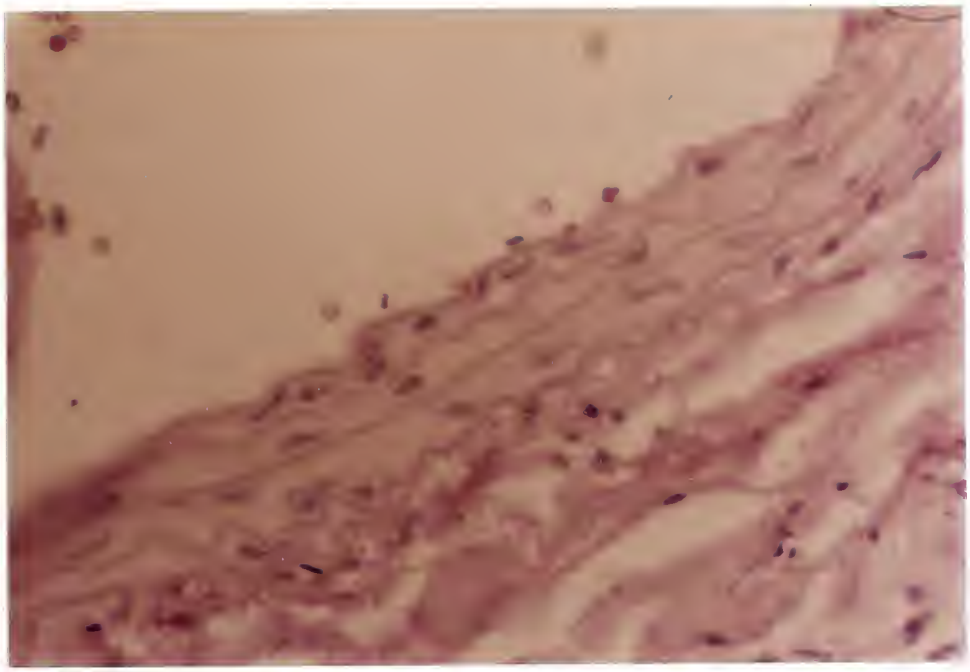
GRAPH 1

CONTROL VS. ASPIRIN: Control and aspirin I/M Ratios graphically displayed. Control data from Days (3+5+7) and (10+14) are combined on this graph. Brackets enclose ± S.E.M.

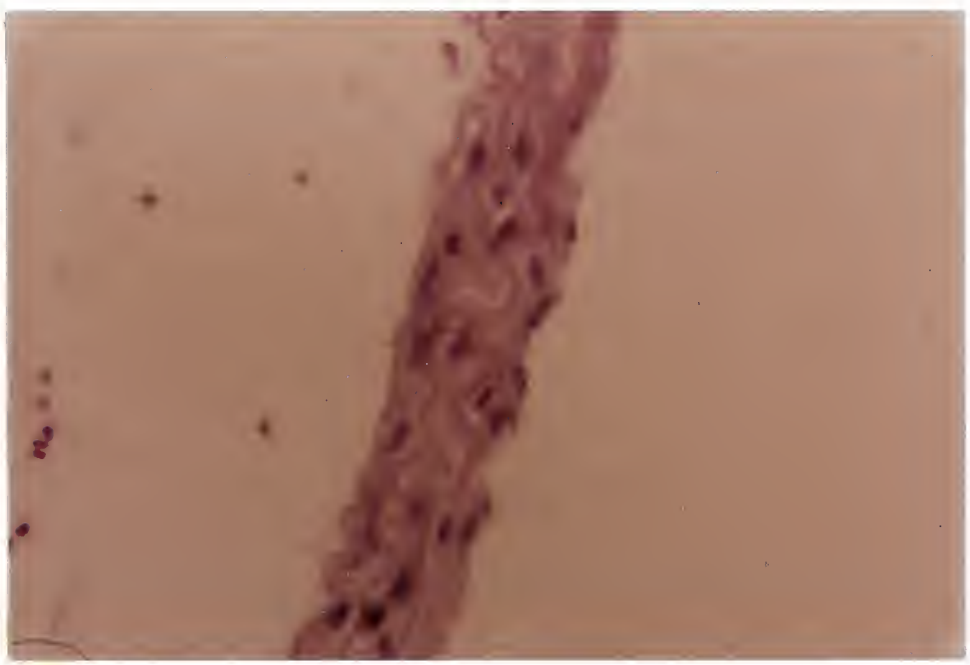


GRAPH 2

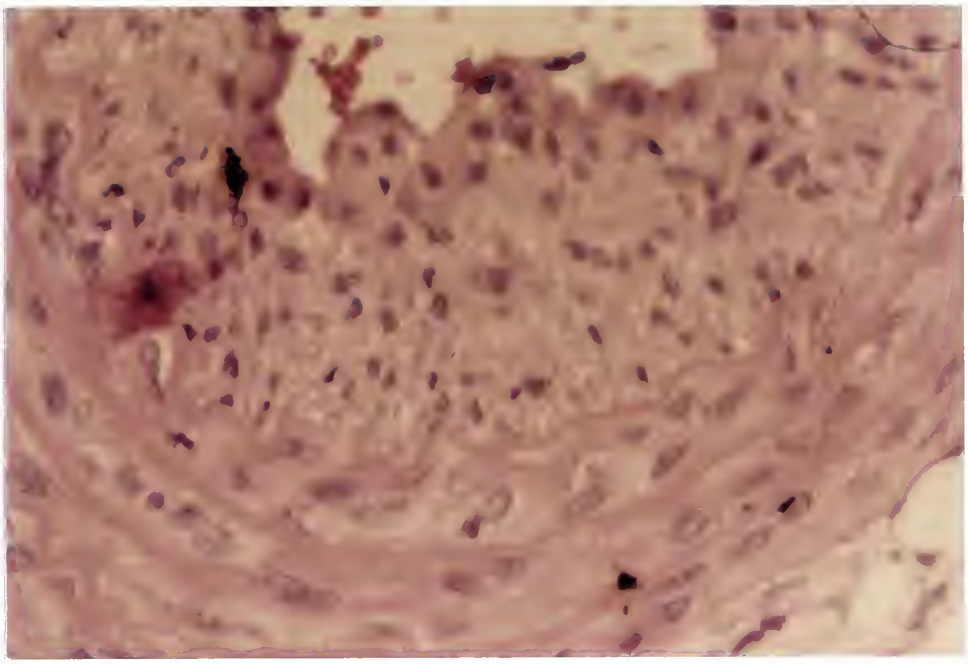
CONTROL VS. WARFARIN: Control and warfarin I/M Ratios graphically displayed. Contol data from Days (3+5+7) and (10+14) are combined on this graph. Brackets enclose ± S.E.M.



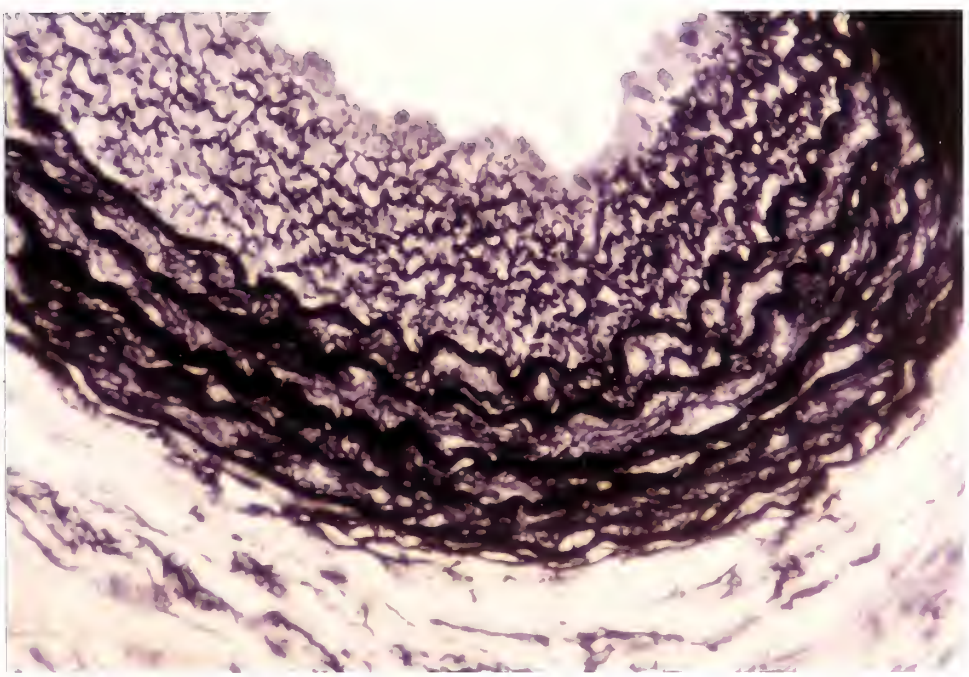
PHOTOGRAPH 1: Day 0 (unoperated) control. One endothelial cell nucleus is visible in the center, lying upon the internal elastic lamina. (Hematoxylin and Eosin, x 450; Lumen - Upper left)



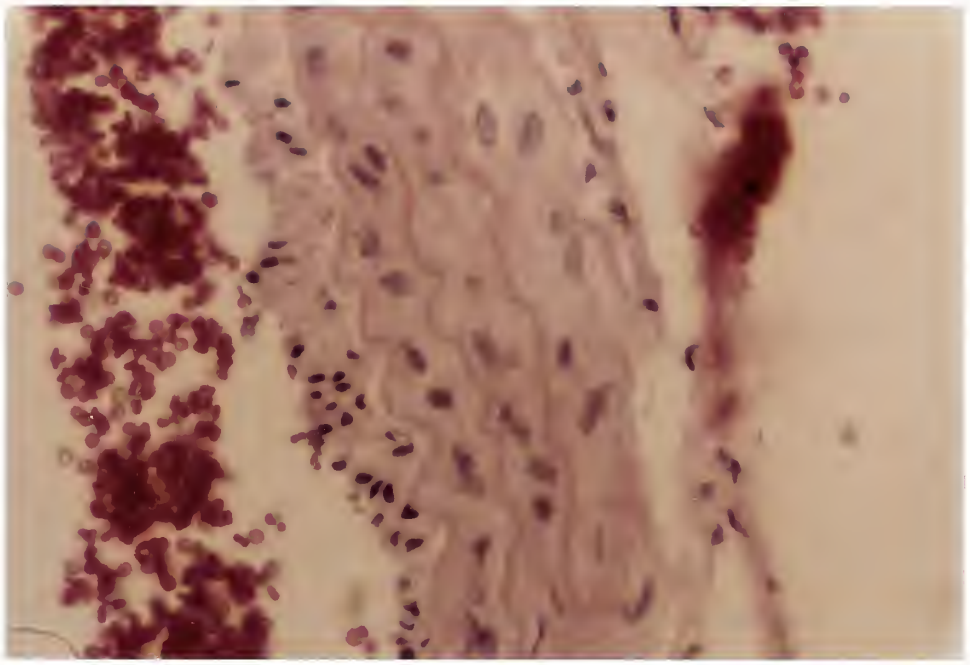
PHOTOGRAPH 2: Day 1 control. Note the absence of endothelial cell nuclei. (Hematoxylin and Eosin, x 450; Lumen - Left half)



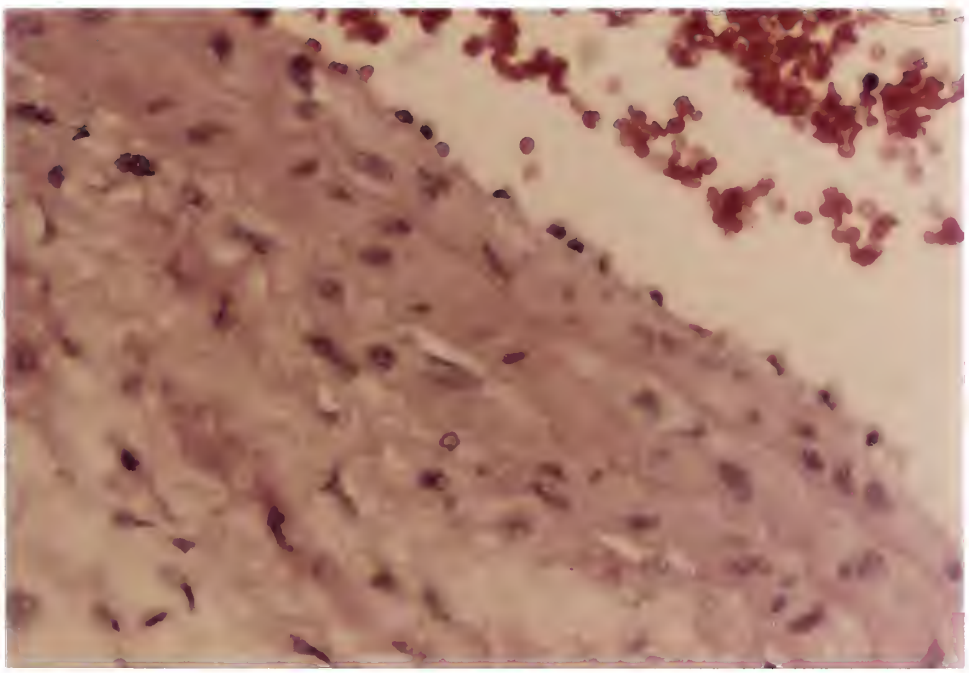
PHOTOGRAPH 3: Day 10 control. Marked myointimal hyperplasia is apparent. Note the medial edema. (Hematoxylin and Eosin, x 450; Lumen - Upper center)



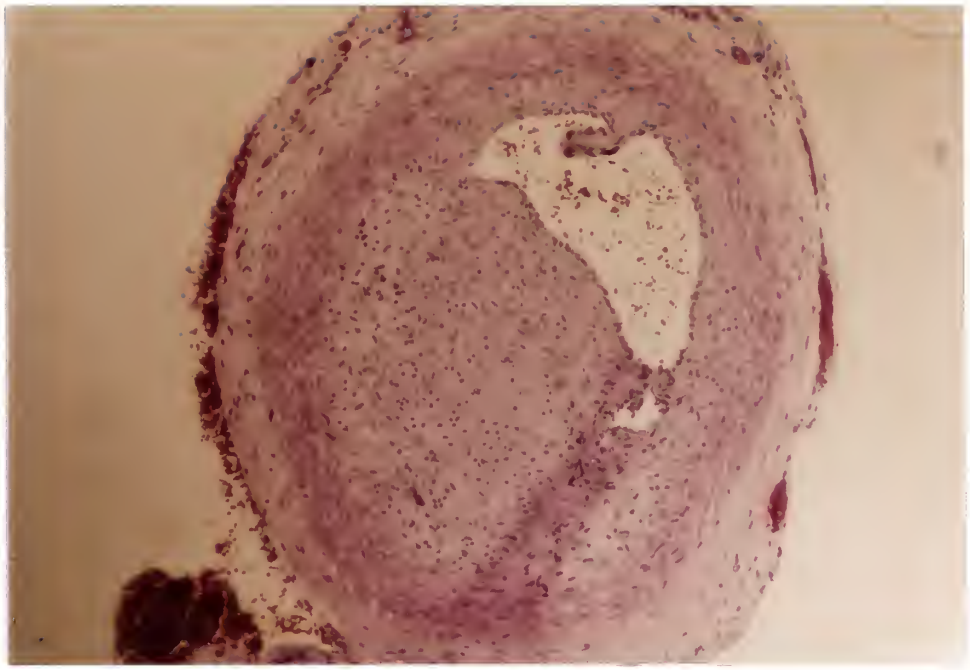
PHOTOGRAPH 4: Day 10 control. Note the copious amount of connective tissue in the neointima. (Elastin-Van Giemsa, x 450; Lumen - Upper center)



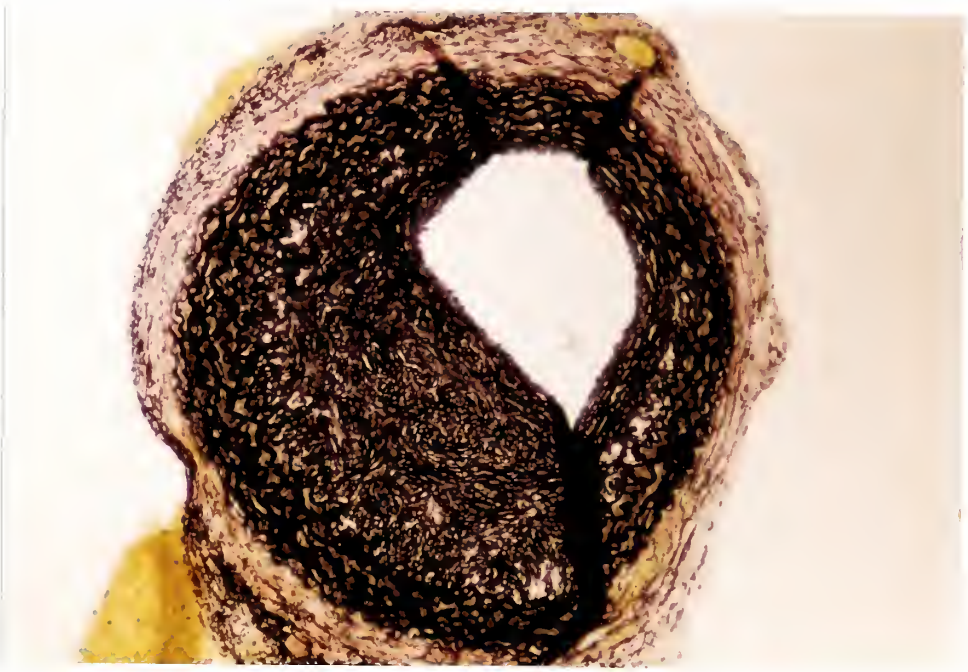
PHOTOGRAPH 5: Day 21 control. The myointimal hyperplasia persists, but has lessened. Medial edema is still evident. (Hematoxylin and Eosin, x 450; Lumen - Left third)



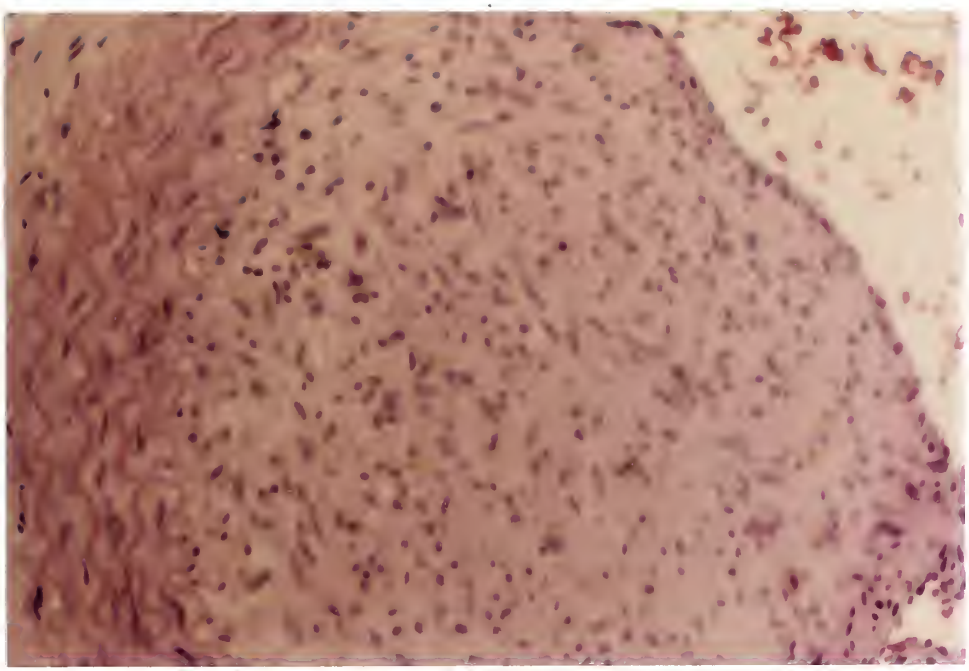
PHOTOGRAPH 6: Day 28 control. Only a minimal amount of intimal thickening is evident. (Hematoxylin and Eosin, x 450; Lumen - Upper right)



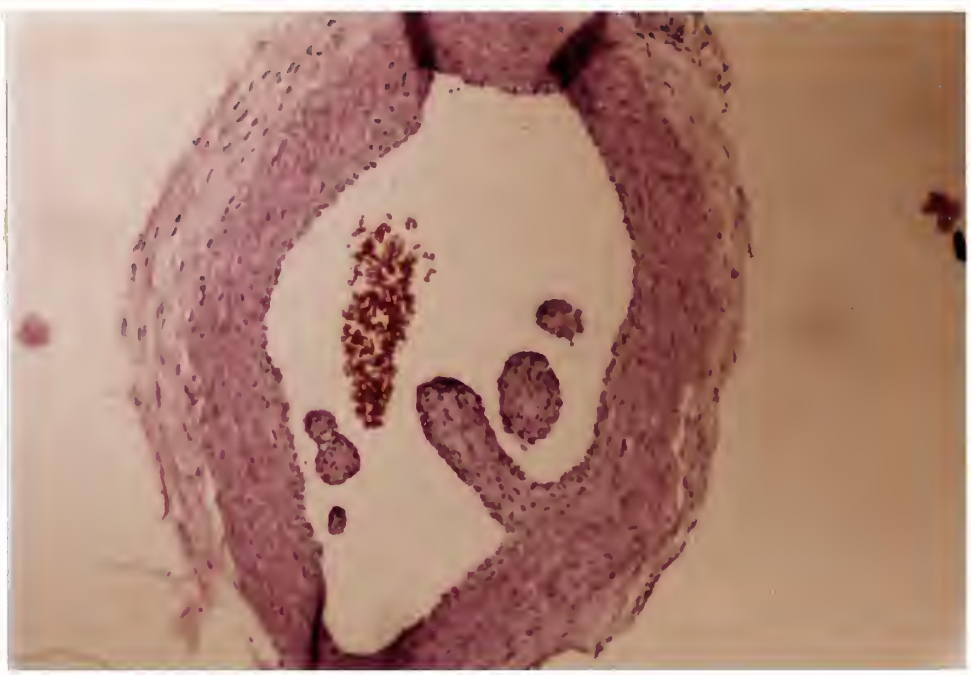
PHOTOGRAPH 7: Day 21 aspirin. The myointimal hyperplasia is massive, nearly occluding the lumen. (Hematoxylin and Eosin, x 100; Lumen - Upper right)



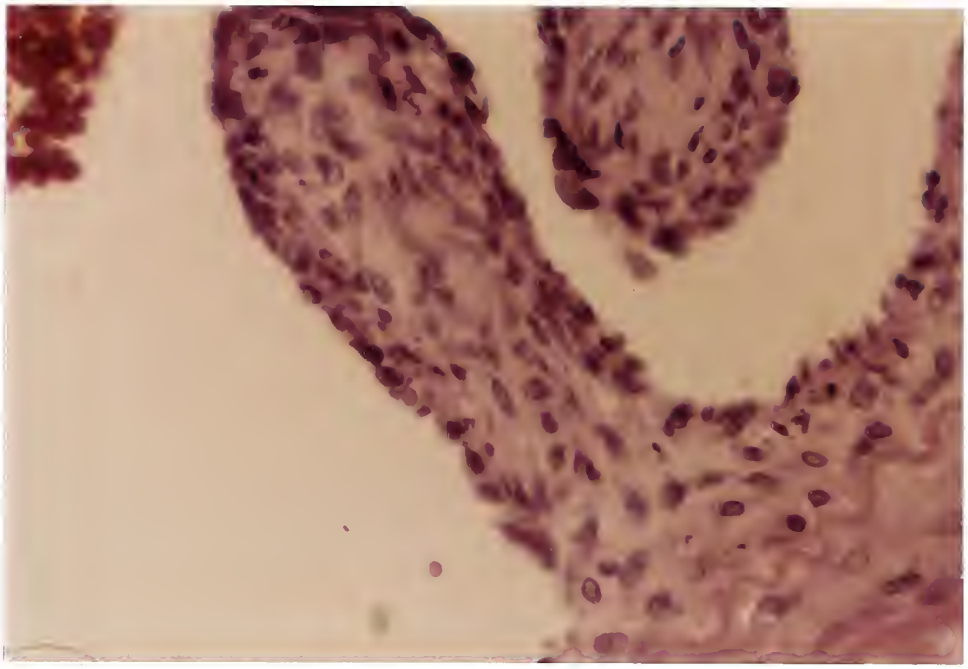
PHOTOGRAPH 8: Day 21 aspirin. Note the eccentricity of the intimal thickening. (Elastin-Van Giemsa, x 100; Lumen - Upper right)



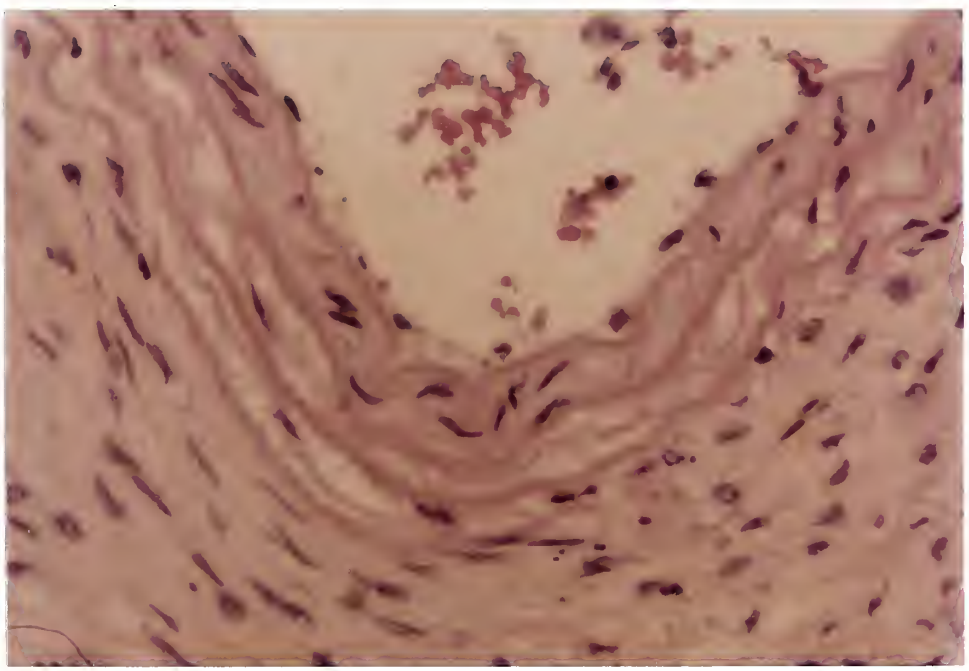
PHOTOGRAPH 9: Day 21 aspirin. A close-up view of the myointimal proliferation seen in Photographs 7 and 8. (Hematoxylin and Eosin, x 450; Lumen - Upper right



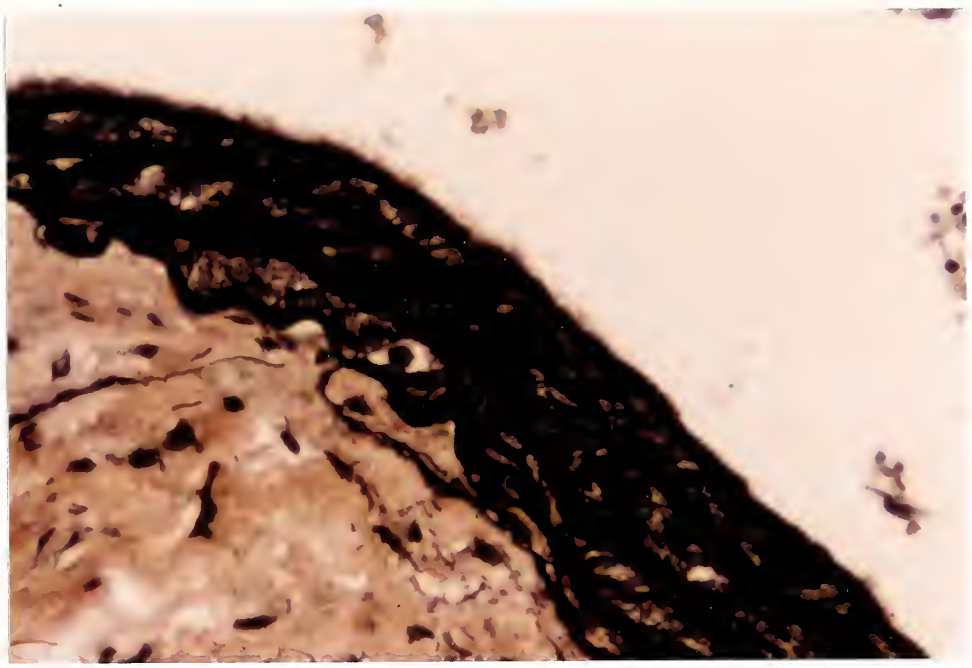
PHOTOGRAPH 10: Day 14 aspirin. A localized proliferation of smooth muscle cells giving the appearance of an intraluminal polyp. (Hematoxylin and Eosin, x 100; Lumen - Center)



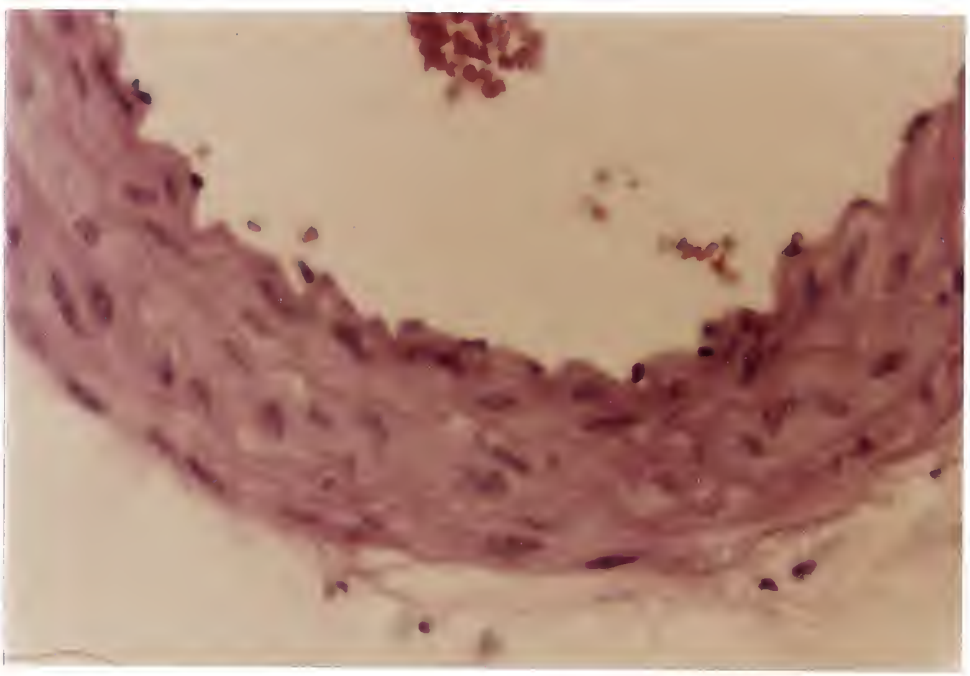
PHOTOGRAPH 11: Day 14 aspirin. A close-up view of the 'polyp' seen in Photograph 10. (Hematoxylin and Eosin, x 450; Lumen - Left two-thirds)



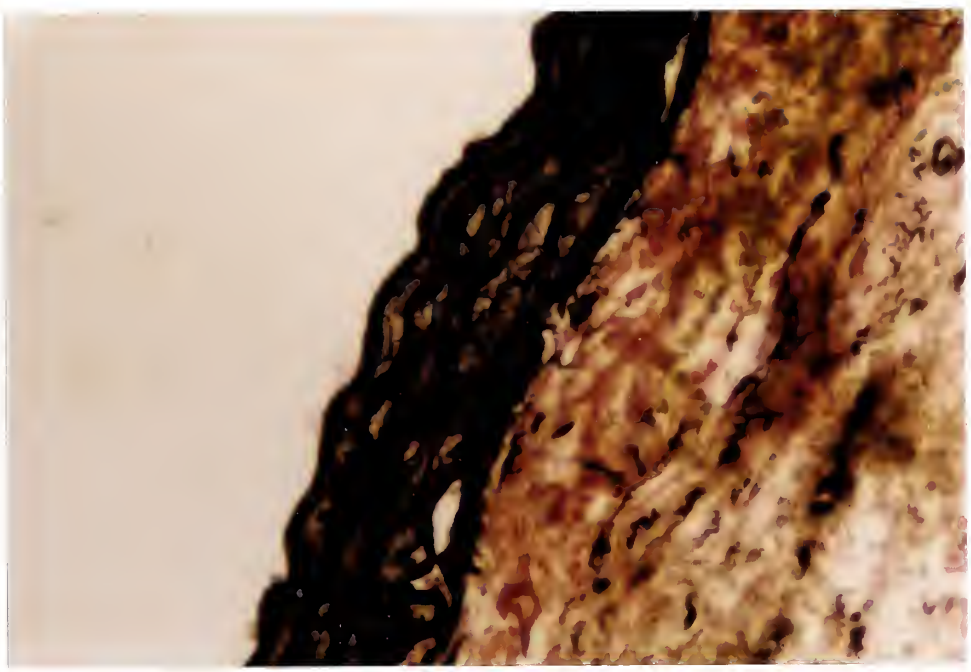
PHOTOGRAPH 12: Day 14 warfarin. Intimal thickening is evident but not especially pronounced. (Hematoxylin and Eosin, x 450; Lumen - Upper center)



PHOTOGRAPH 13: Day 14 warfarin. Only a minimal amount of intimal connective tissue is seen. (Elastin-Van Giemsa, x 450; Lumen - Upper right)

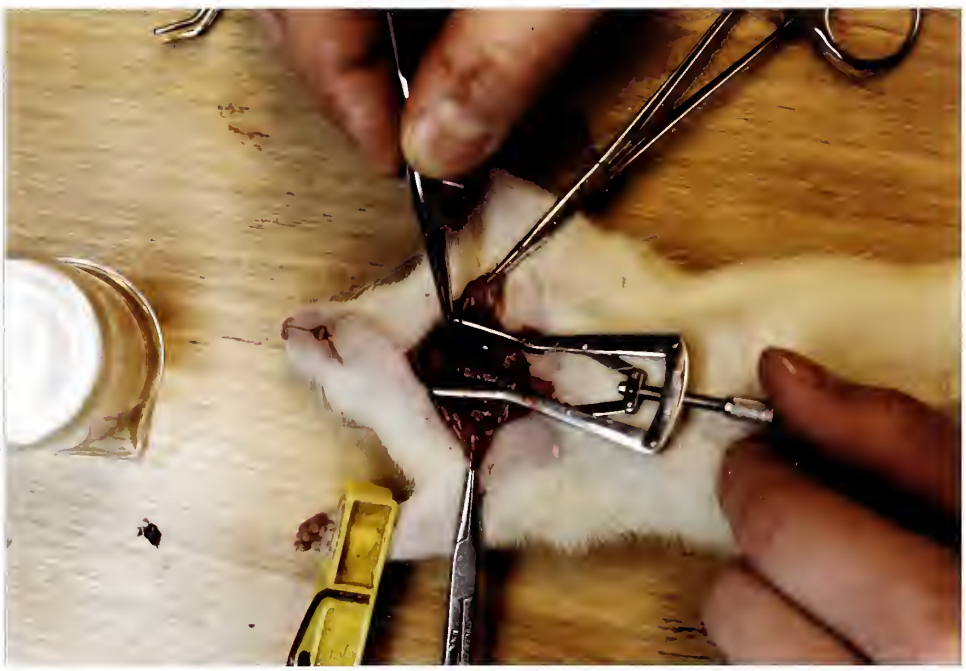


PHOTOGRAPH 14: Day 28 warfarin. The intima is normal in appearance, showing no residual hyperplasia. (Hematoxylin and Eosin, x 450; Lumen - Upper center)

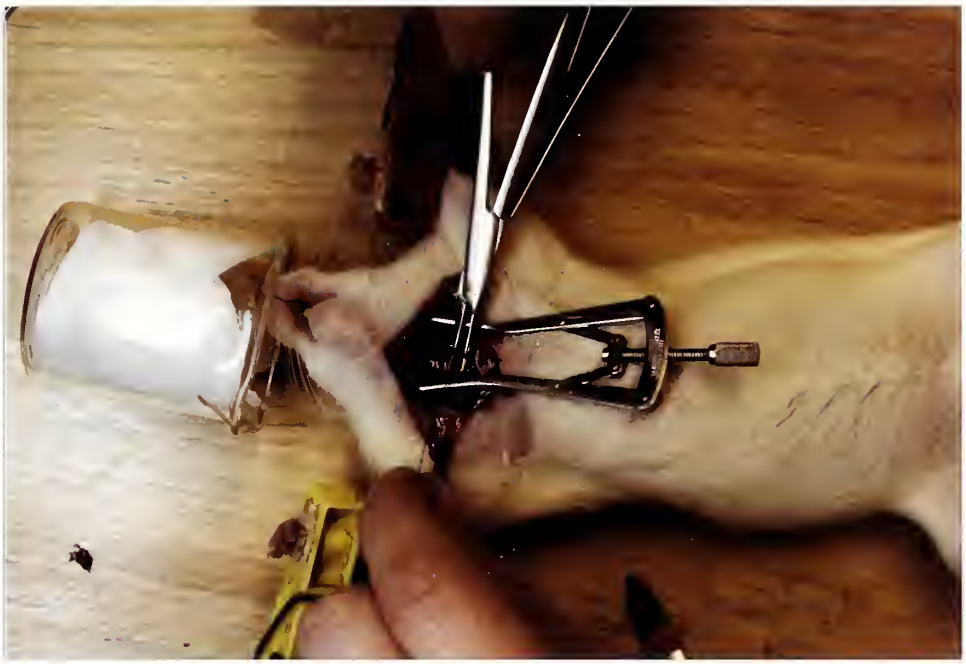


PHOTOGRAPH 15: Day 28 warfarin. Note the absence of intimal connective tissue. (Elastin-Van Giemsa, x 450; Lumen - Left third)

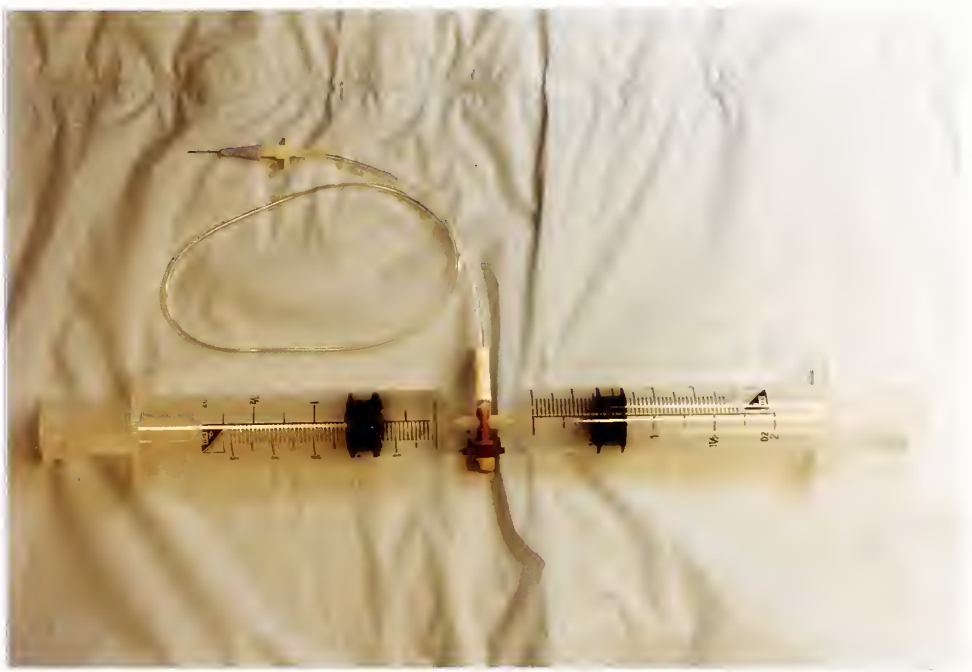
PHOTOGRAPH 16: The set of instruments and materials used during the operative procedure.



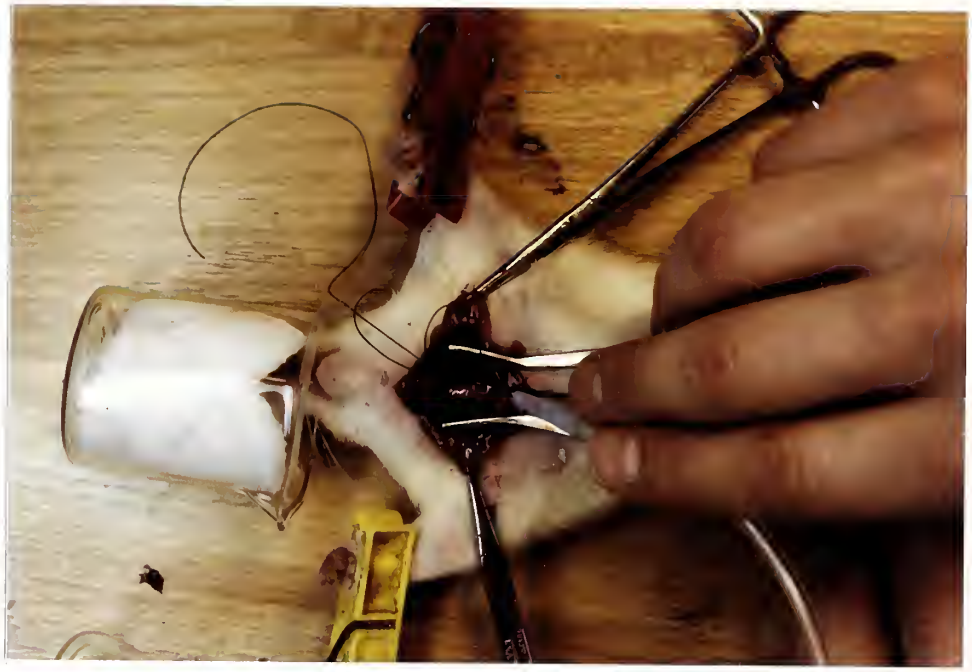
PHOTOGRAPH 17: Exposed cervical structures. The hemostats are retracting the submaxillary glands. The self-retaining retractor is separating the sternohyoid muscles. The tip of the forceps is on the left common carotid artery. The glistening structure in the midline is the trachea.



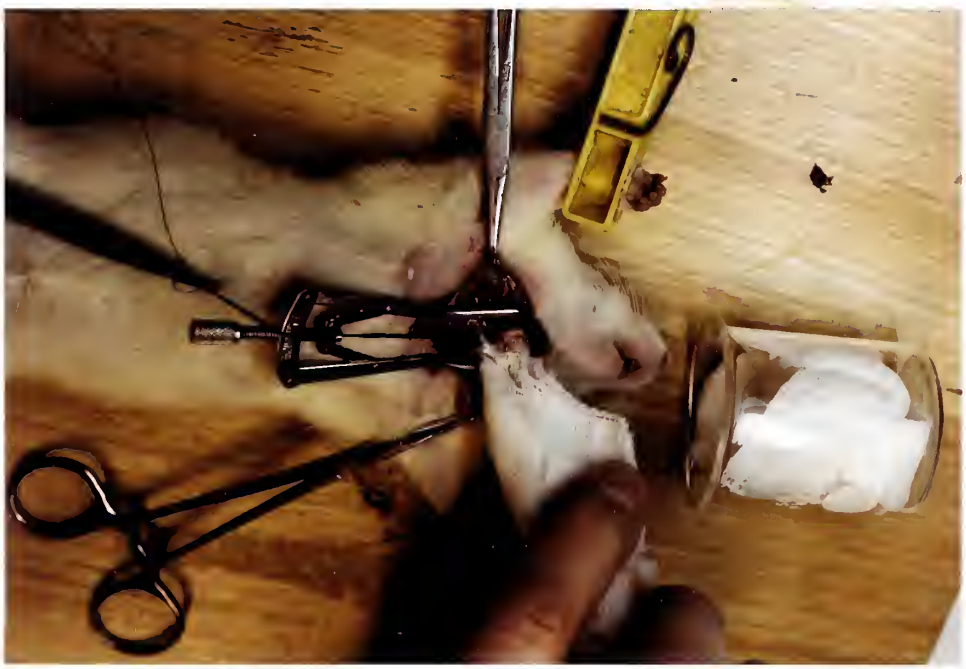
PHOTOGRAPH 18: The first ligature being threaded underneath the left common carotid artery. The artery is sitting on top of the hemostat just left of the trachea.



PHOTOGRAPH 19: The needle-connecting tube-stopcock-syringe arrangement. The syringe on the left contains normal saline. The syringe on the right contains room air. The needle is 30-gauge.



PHOTOGRAPH 20: The desiccation procedure. The needle tip is in the vessel lumen. The vessel is manipulated via tension on the proximal (inferior) ligature. The distal ligature is seen exiting the wound superiorly.



PHOTOGRAPH 21: Hemostasis is acheived with gentle pressure from a 4 x 4 gauze sponge.

PHOTOGRAPH 22: The finished product. Approximately two-thirds of the rats undergoing the procedure were left with a left Horner's Syndrome. Otherwise complete recovery was acheived within twenty-four hours post-operatively.

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